Exploring Redox Biology in physiology and disease

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Chapter 9

Urinary excretion of sulfur metabolites and risk of cardiovascular events and all-cause mortality in the general population

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Abstract

Aims

Thiosulfate and sulfate are metabolites of hydrogen sulfide (H_2S), a gaseous signaling molecule with cardiovascular protective properties. Moreover, sulfate itself is essential to life. Urinary thiosulfate and sulfate excretion are associated with favorable disease outcome in high-risk patient groups. This study aimed to investigate the relationship between urinary excretion of these sulfur metabolites and risk of cardiovascular (CV) events and all-cause mortality in the general population.

Results

A total of 6839 subjects of the Prevention of Renal and Vascular End-stage Disease (PREVEND) study were followed prospectively. At baseline, 24-h urinary excretion of thiosulfate and sulfate were determined. Median urinary thiosulfate and sulfate excretion were 1.27 (ICR 0.89-2.37) µmol/24 h and 15.7 (IQR 12.0-20.3) mmol/24 h, respectively. Neither thiosulfate, nor sulfate excretion showed an independent association with risk of CV events. Sulfate, but not thiosulfate, was found to be inversely associated with risk of all-cause mortality, independent of potential confounders (hazard ratio 0.73 (95% confidence interval 0.63-0.84), $P<0.001$). This association appeared most pronounced for normolipidemic subjects ($P_{interaction}= 0.019$).

Innovation

The strong association between sulfate excretion and mortality in a large, general population based cohort emphasizes the (patho)physiological importance of sulfate or its precursor H_2S. Sulfate excretion holds promise as a marker of physiological disturbance and may even serve as a target for nutritional intervention.

Conclusion

In conclusion, urinary sulfate, but not thiosulfate excretion is inversely associated with all-cause mortality in the general population. Further research is warranted to unravel the significance of urinary sulfate excretion in physiology and disease.

1 Introduction

In the general population, cardiovascular disease (CVD) is the leading cause of death worldwide(28). Despite evolving efforts to control known risk factors(39), the incidence of CVD continues to increase, warranting the exploration of novel pathways for cardiovascular (CV) risk reduction.

This study focuses on the urinary excretion of the sulfur metabolites thiosulfate ($S_2O_3^{2-}$) and sulfate (SO_4^{2-}). These metabolites arise from the oxidation of sulfur containing amino acids (SAAs) in the so-called transsulfuration pathway(15). Interestingly, one of the intermediates in this process is hydrogen sulfide (H_2S), a gaseous signaling molecule with various protective properties(41), including the potential to counteract CVD(30). Indeed, several preclinical studies have convincingly shown H_2S to play a role in CV protection. In mice, deficiency of cystathionine γ-lyase (CSE) - one of the H_2S producing enzymes - and consequent decreased systemic H_2S levels are accompanied by hyperhomocysteinemia, hypertension and impaired vasorelaxation(53). Also, exogenous administration of H_2S or H_2S donors has been shown to offer protection in experimental models of atherosclerosis(3, 23, 30), cardiac injury(36, 37, 42), renal disease(17, 37), and stroke(35, 50).

Furthermore, sulfate itself is a dietary constituent and involved in various (patho)physiological processes(6, 22). Through sulfate conjugation or sulfation, it is responsible for the biotransformation and detoxification of many endogenous and exogenous substances(6, 8, 22). Sulfate may thereby directly contain disease development. Conversely, sulfated toxic intermediates, including indoxyl sulfate and p-cresyl sulfate, have been implicated in carcinogenesis, as well as heart failure events(2, 10, 49). As sulfation is generally considered to increase hydrophilicity and thereby to promote renal elimination of its targets(5), variations in urinary sulfate excretion may reflect the need for sulfate-mediated detoxification. However, the way in which sulfate conjugation is related to the toxicity of these specific compounds is yet unknown.

Previous studies of high-risk populations have shown that urinary excretion of thiosulfate and sulfate are associated with a favorable CV risk profile and survival in renal transplant recipients(45) and preservation of renal function in patients with diabetes(1, 46). In addition, our group has recently shown both urinary excretion and clearance of sulfate to be associated with a decreased rehospitalization rate and increased patient survival in chronic heart failure (CHF)(18). The apparent link to CV (patho)physiology led us to hypothesize that urinary excretion of sulfur metabolites is inversely associated with CV events and mortality in the general population, as it is in high-risk patient groups. Therefore, the aim of the present study was to determine the association of urinary thiosulfate and sulfate excretion with risk of CV events and all-cause mortality in a large cohort of individuals from the general population.

2 Results[3]

2.1 Cohort characteristics

Participants had a median 24-h urinary thiosulfate excretion of 1.27 (0.89-2.37) µmol/24 h and a median 24-h urinary sulfate excretion of 15.7 (12.0-20.3) mmol/24 h and a. Baseline characteristics are presented in Table 1, overall and categorized by gender-stratified quintiles of sulfate excretion. The mean age was 53.4 ± 12.1 years and 50% of the subjects (*n*=3420) were female. 24-h urinary urea excretion, which provides a rough estimate of dietary protein intake, rises with every quintile of urinary sulfate excretion. Subjects in the highest quintile were younger and had a larger body surface area (BSA). Systolic blood pressure and heart rate were lower in this group. Subjects in the lowest quintile of urinary sulfate excretion more often had a history of CV events, which was accompanied by the highest use of anti-hypertensive and lipid lowering treatment. Also, this quintile contained the most current smokers and its subjects had the lowest estimated glomerular filtration rate (eGFR).

2.2 Factors associated with urinary excretion of thiosulfate and sulfate

Univariable and multivariable linear regression analyses identified gender, BSA, diabetes and current smoking as potential confounders for 24-h urinary thiosulfate excretion (Tab. 2). The same analyses identified gender, age, BSA, history of CVD, current smoking and consumption of more than 1 alcoholic beverage per day as potential confounders for daily urinary sulfate excretion (Tab. 3).

As shown in Table 4, 24-h urinary thiosulfate excretion is positively associated with systolic and diastolic blood pressure, anti-diabetic treatment, glucose, eGFR and 24-h urinary albumin and sulfate excretion, and inversely associated with high-density lipoprotein (HDL). The same table shows that 24-h urinary sulfate excretion is positively associated with diastolic blood pressure, glucose, eGFR and 24-h urinary albumin and thiosulfate excretion, and inversely associated with heart rate, anti-hypertensive and lipid lowering treatment, HDL and high sensitivity C-reactive protein (hs-CRP).

2.3 Urinary excretion of thiosulfate and sulfate and risk of cardiovascular events

In the course of follow-up for 8.2 (7.7-8.8) years 504 CV events were recorded. 239 subjects (47.4%) suffered from ischemic heart disease of whom 36 (15.1%) died. A cerebrovascular event occurred in 111 subjects (22%), of whom 4 (3.6%) died.

Crude Cox proportional hazards analyses showed a significant inverse association of 24-h urinary sulfate, but not thiosulfate excretion with risk of CV events (Tab. 5, model 1 for 24-h urinary sulfate excretion, hazard ratio (HR) per doubling 0.87 (0.76-0.99), P=0.039). However, this association lost its statistical significance by adjustment for potential confounding factors (gender, age, BSA, history of cardiovascular disease, diabetes, current smoking and consumption of more than 1 alcoholic beverage per day; Tab. 5, model 2 for 24-h urinary sulfate excretion, HR per doubling 0.89 (0.77-1.03), P=0.107).

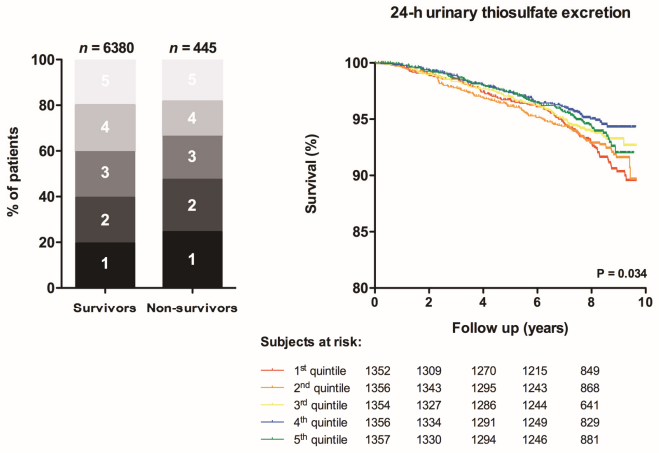
[3] Tables can be found at the end of this section.

2.4 Urinary excretion of thiosulfate and sulfate and risk of all-cause mortality

During follow-up a total of 445 subjects (6.5%) died. Of these, 120 (27.0%) died of a cardiovascular cause.

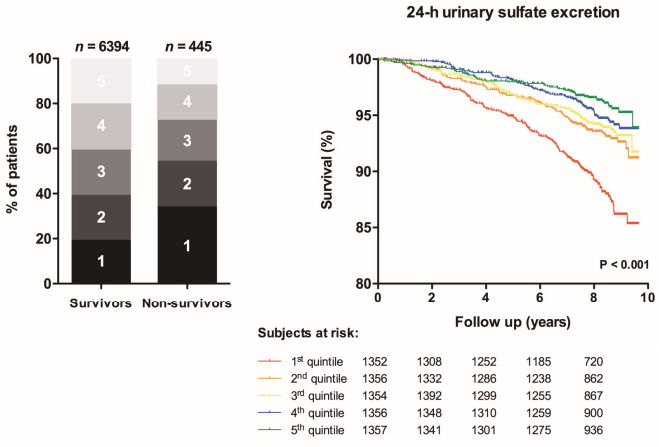
Most deaths occurred in the lowest quintiles of 24-h urinary thiosulfate and sulfate excretion (109 (8.0%), log rank test, P=0.036 and 151 (11%), log rank test, P<0.001, respectively). The corresponding Kaplan-Meier plots are shown in Figure 1 and 2. These figures also demonstrate the differential distribution of quintiles of thiosulfate and sulfate excretion among survivors and non-survivors ($\chi 2$ test, P=0.011, Fig. 1 and $\chi 2$ test, P<0.001, Fig. 2, respectively), with overrepresentation of the lowest quintile, particularly of sulfate excretion, in the non-survivors population.

Figure 1: Survival distributions of urinary thiosulfate excretion quintiles



A. *Gender-stratified quintiles of 24-h urinary thiosulfate excretion are differentially distributed among survivors and non-survivors with overrepresentation of the lowest quintile in the non-survivors population (χ^2 test, P=0.011).* ***B.*** *Kaplan-Meier plot with log-rank test for survival showing the highest mortality rate for the lowest quintile of thiosulfate excretion (P=0.034).*

Figure 2: Survival distributions of urinary sulfate excretion quintiles



A. *Gender-stratified quintiles of 24-h urinary sulfate excretion are differentially distributed among survivors and non-survivors with overrepresentation of the lowest quintile in the non-survivors population (χ^2 test, P<0.001).* ***B.*** *Kaplan-Meier plot with log-rank test for survival showing the highest mortality rate for the lowest quintile of sulfate excretion (P<0.001).*

Despite the significant difference in the survival distributions for quintiles of thiosulfate excretion determined by the log-rank test, Cox proportional hazards analysis showed no association between 24-h urinary thiosulfate excretion and all-cause mortality (Tab. 6, model 1 for 24-h urinary thiosulfate excretion, HR per doubling 0.93 (0.86-1.01), P=0.100).

For 24-h urinary sulfate excretion Cox proportional hazards analysis did show a significant inverse association with all-cause mortality (Tab. 6, model 1 for 24-h urinary sulfate excretion, HR per doubling 0.65 (0.57-0.74), P<0.001), which remained significant after adjustment for potential confounders (Tab. 6, model 2 for 24-h urinary sulfate excretion, HR per doubling 0.73 (0.63-0.84), P<0.001), and further adjustment for 24-h urinary urea excretion (HR per doubling 0.66 (0.54-0.80), P<0.001). Crude Cox regression also showed an association of urinary sulfate excretion with death of a CV cause (HR per doubling 0.64 (0.51-0.82), P<0.001). However, after correction for potential confounders, this association was no longer significant (HR per doubling 0.77 (0.58-1.02), P=0.070).

Restricted cubic splines showed no significant deviances from linear associations with all-cause mortality for either 24-h urinary thiosulfate or sulfate excretion (Fig. 3, $P_{nonlinearity}$=0.08 and 0.10, respectively).

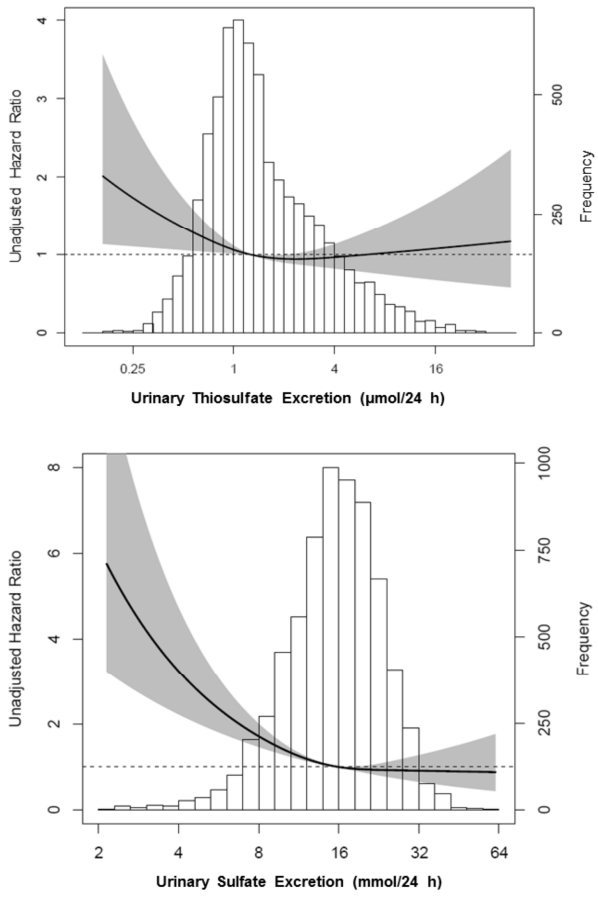


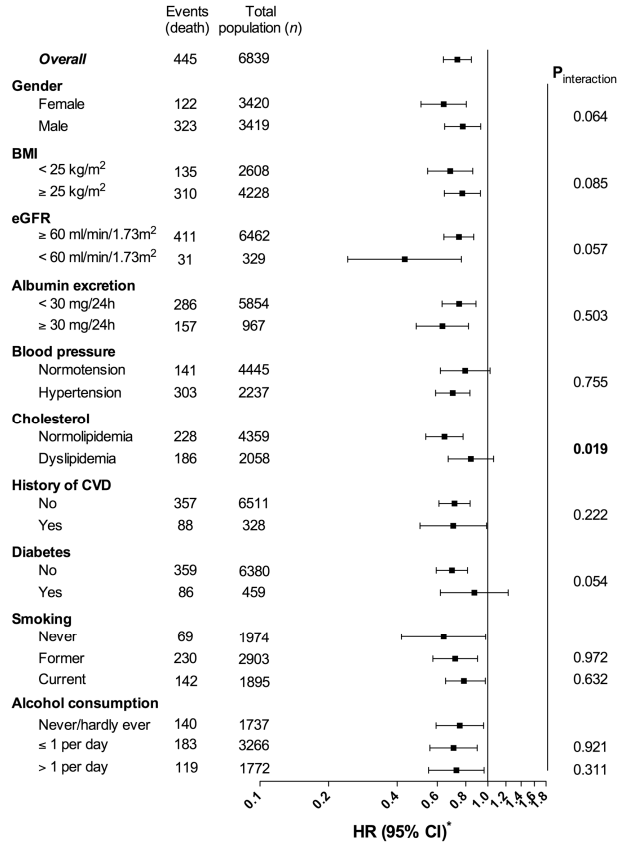
Figure 3: No deviances from linear associations with all-cause mortality for urinary thiosulfate and sulfate excretion

Unadjusted associations estimated by Cox proportional hazards analyses based on restricted cubic splines show no deviances from linear associations for 24-h urinary thiosulfate and sulfate excretion ($P_{nonlinearity}$=0.08 and 0.10, respectively). Median 24-h urinary thiosulfate and sulfate excretion (1.27 µmol and 15.7 mmol/24 h, respectively) are the reference standards. Grey area indicate 95% confidence intervals.

Stratified analyses of the association between 24-h urinary sulfate excretion and all-cause mortality showed consistent hazard ratios across various subgroups, except for those stratified by cholesterol (normo- vs. dyslipidemia) (Fig. 4, $P_{interaction}$=0.019). Stratification by renal function (eGFR ≥ 60 vs. < 60 ml/min) and the presence of diabetes (no diabetes vs.

diabetes) resulted in borderline significant differences between groups (Fig. 4, $P_{interaction}$=0.057 and $P_{interaction}$=0.054, respectively). The corresponding hazard ratios were lower for subjects with normolipidemia, those with impaired renal function (eGFR < 60 ml/min) and those without diabetes.

Figure 4: Associations between urinary sulfate excretion and all-cause mortality in different subgroups



Hazard ratios for all-cause mortality in various subgroups, consistently showing positive associations between 24-h urinary sulfate excretion and survival. Only cholesterol levels (normo- vs. dyslipidemia) show significant interaction ($P_{interaction}$=0.019). Stratifications by renal function (eGFR ≥ 60 vs. < 60 ml/min) and the presence of diabetes (no diabetes vs. diabetes) reveal borderline significant differences between groups ($P_{interaction}$=0.057 and $P_{interaction}$=0.054, respectively). Corresponding hazard ratios are lower for subjects with normolipidemia, subjects with impaired renal function (eGFR < 60 ml/min) and subjects without diabetes.

**After adjustment for potential confounders (sex, age, BSA, history of cardiovascular disease, diabetes, current smoking and consumption of more than 1 alcoholic beverage per day)*

2.5 Causal path analysis

In causal path analyses, following adjustment for potential confounders, the association of 24-h urinary sulfate excretion and all-cause mortality was further adjusted for hemodynamic parameters (systolic and diastolic blood pressure, heart rate and anti-hypertensive treatment), lipid profile (total cholesterol, HDL, triglycerides and lipid lowering treatment), hs-CRP and eGFR. None of these adjustments affected the statistical significance of the association between 24-urinary sulfate excretion and all-cause mortality (Tab. 7, model 2-5, all P<0.001).

Tables

Table 1. Baseline characteristics by gender-stratified quintiles of 24-h urinary sulfate

	Overall (*n*=6839)	**1st quintile (*n*=1367)**	**2nd quintile (*n*=1368)**	**3rd quintile (*n*=1368)**	**4th quintile (*n*=1368)**	**5th quintile (*n*=1368)**	**P-value**
24-h urinary sulfate, mmol/24 h*	15.7 [12.0-20.3]	♀: < 10.0 ♂: < 12.8	♀: 10.0-12.8 ♂: 12.8-16.2	♀: 12.8-15.3 ♂: 16.2-19.7	♀: 15.4-18.6 ♂: 19.7-23.9	♀: > 18.6 ♂: > 23.9	
Demographics							
Female, *n* (%)	3420 (50)	684 (50)	684 (50)	684 (50)	684 (50)	684 (50)	1
Age, years	53.4 ± 12.1	55.7 ± 12.9	54.8 ± 12.6	53.5 ± 12.0	52.1 ± 11.6	50.9 ± 10.6	**<0.001**
BSA, m^2	1.93 ± 0.20	1.88 ± 0.20	1.9 ± 0.19	1.9 ± 0.18	2.0 ± 0.19	2.0 ± 0.21	**<0.001**
Systolic blood pressure, mmHg	126 ± 18.9	128 ± 20.5	127 ± 19.5	126 ± 18.6	125 ± 18.5	125 ± 17.2	**<0.001**
Diastolic blood pressure, mmHg	73.4 ± 9.10	73.6 ± 9.1	73.8 ± 9.1	73.5 ± 9.4	73 ± 9.1	73.3 ± 8.8	0.259
Heart rate, bpm	68.4 ± 10.1	69.4 ± 11.1	68.9 ± 10.2	67.9 ± 10.0	68.1 ± 9.5	68.0 ± 9.9	**<0.001**
History of cardiovascular disease, *n* (%)	316 (4.8)	94 (7)	62 (5)	71 (5)	49 (4)	40 (3)	**<0.001**
Diabetes, *n* (%)	440 (7)	92 (7)	84 (6)	74 (6)	89 (7)	101 (8)	0.264
Smoking status, *n* (%)							**<0.001**
Never	1974 (29)	337 (25)	395 (29)	417 (31)	403 (30)	422 (31)	
Former	2903 (43)	460 (34)	576 (43)	594 (44)	614 (45)	659 (49)	
Current	1895 (28)	555 (41)	381 (28)	348 (26)	342 (25)	269 (20)	
Alcohol consumption, *n* (%)							**<0.001**
Never/hardly ever	1737 (26)	437 (32)	409 (30)	304 (22)	298 (22)	289 (21)	
Up to 1/day	3266 (48)	583 (43)	620 (46)	701 (52)	690 (51)	672 (50)	
> 1/day	1772 (26)	333 (25)	323 (24)	354 (26)	371 (27)	391 (29)	
Medication							
Anti-hypertensive treatment, *n* (%)	1408 (24)	334 (27)	319 (26)	276 (23)	236 (20)	243 (21)	**<0.001**
Lipid lowering treatment, *n* (%)	590 (10)	165 (14)	129 (11)	115 (10)	99 (8)	82 (7)	**<0.001**

Anti-diabetic treatment, *n* (%)	233 (4)	57 (5)	34 (3)	39 (3)	47 (4)	56 (5)	0.052
Laboratory measurements							
Total cholesterol, mmol/l	5.4 ± 1.1	5.4 ± 1.1	5.5 ± 1.0	5.4 ± 1.0	5.5 ± 1.0	5.5 ± 1.1	0.106
HDL, mmol/l	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	0.230
Triglycerides, mmol/l*	1.1 [0.8-1.6]	1.2 [0.9-1.7]	1.1 [0.8-1.6]	1.1 [0.8-1.6]	1.1 [0.8-1.6]	1.1 [0.8-1.6]	**<0.001**
Glucose, mmol/l*	4.8 [4.4-5.3]	4.8 [4.4-5.4]	4.8 [4.4-5.3]	4.8 [4.4-5.3]	4.8 [4.4-5.4]	4.8 [4.5-5.4]	**0.010**
hs-CRP, mg/l*	1.4 [0.6-3.1]	1.5 [0.7-3.3]	1.4 [0.7-3.3]	1.3 [0.6-3.1]	1.3 [0.6-2.7]	1.4 [0.6-3.1]	**0.003**
eGFR, ml/min per 1.73 m^2	85.8 ± 14.3	84.1 ± 14.9	84.3 ± 15.1	85.9 ± 14.2	87.0 ± 13.5	87.4 ± 13.5	**<0.001**
24-h urinary albumin, mg/24 h*	8.7 [6.0-16]	7.7 [5.1-14.9]	7.9 [5.7-15.1]	8.7 [6.1-16.0]	9.1 [6.4-16.3]	9.9 [6.9-18.8]	**<0.001**
24- h urinary thiosulfate, µmol/24 h*	1.27 [0.89-2.37]	1.04 [0.73-1.56]	1.15 [0.83-1.87]	1.29 [0.9-2.3]	1.44 [1.0-3.0]	1.76 [1.1-3.7]	**<0.001**
24-h urinary urea, mmol/24 h	367 ± 125	245 ± 75.6	315 ± 70.7	360 ± 78.2	411 ± 83.7	504 ± 127	**<0.001**

*Normally distributed continuous data are presented as mean ± SD *Skewed data are presented as median (IQR)*
BSA; body surface area, bpm; beats per min HDL; high-density lipoprotein, hs-CRP; high sensitivity C-reactive protein, eGFR; estimated glomerular filtration rate, SD; standard deviation, IQR; interquartile range

Table 2: Univariable and multivariable linear regression analyses of 24-h urinary thiosulfate excretion and potential confounders

24-h urinary thiosulfate excretion*				
	Univariable regression		Multivariable regression	
	Coefficient	P-value	Coefficient	P-value
Demographics				
Female gender	-0.116	**<0.001**	-0.043	**0.005**
Age	-0.019	0.107		
BSA	0.164	**<0.001**	0.116	**<0.001**
History of cardiovascular disease	0.024	0.056		
Diabetes	0.072	**<0.001**	0.056	**<0.001**
Current smoking	-0.172	**<0.001**	-0.157	**<0.001**
> 1 alcoholic consumption/day	0.008	0.51		

**Skewed data, normalized by logarithmic transformation*
BSA; body surface area, BMI; body mass index

Table 3: Univariable and multivariable linear regression analyses of 24-h urinary sulfate excretion and potential confounders

24-h urinary sulfate excretion*				
	Univariable regression		Multivariable regression	
	Coefficient	P-value	Coefficient	P-value
Demographics				
Female gender	-0.266	**<0.001**	-0.026	**0.045**
Age	-0.110	**<0.001**	-0.046	**<0.001**
BSA	0.339	**<0.001**	0.043	**0.008**
History of cardiovascular disease	-0.033	**0.007**		
Diabetes	0.017	0.160		
Current smoking	-0.156	**<0.001**	-0.076	**<0.001**
> 1 alcoholic consumption/day	0.064	**<0.001**	0.045	**<0.001**

**Skewed data, normalized by logarithmic transformation*
BSA; body surface area, BMI; body mass index

Table 4: Univariable linear regression analyses of 24-h urinary thiosulfate and sulfate excretion and potential associated factors, adjusted for confounders

24-h urinary thiosulfate excretion*				
	Univariable regression		Adjusted for confounders	
	Coefficient	P-value	Coefficient	P-value
Demographics				
Systolic blood pressure	0.027	**0.027**	-0.005	0.693
Diastolic blood pressure	0.046	**<0.001**	0.054	**<0.001**
Heart rate	-0.015	0.213	-0.084	**<0.001**
Medication				
Anti-hypertensive treatment	0.015	0.236	-0.051	**<0.001**
Lipid lowering treatment	-0.003	0.792	-0.052	**<0.001**
Anti-diabetic treatment	0.058	**<0.001**	0.012	0.358
Laboratory measurements				
Total cholesterol	-0.023	0.054	-0.014	0.263
HDL	-0.044	**<0.001**	-0.80	**<0.001**
Triglycerides*	0.092	0.762	-0.004	0.774
Glucose*	0.070	**<0.001**	0.064	**<0.001**
hs-CRP*	-0.005	0.685	-0.072	**<0.001**
eGFR	0.071	**<0.001**	0.199	**<0.001**
24-h urinary albumin*	0.042	**<0.001**	0.124	**<0.001**
24-h urinary thiosulfate	-	**-**	0.289	**<0.001**
24-h urinary sulfate*	0.289	**<0.001**	-	**-**

**Skewed data, normalized by logarithmic transformation*
HDL; high-density lipoprotein, hs-CRP; high sensitivity C-reactive protein, eGFR; estimated glomerular filtration rate

Table 5: Cox proportional hazard models of the association of 24-h urinary sulfate and thiosulfate and sulfate excretion and potential confounders with cardiovascular events

24-h urinary thiosulfate excretion (µmol/24 h)						
		Gender-stratified quintiles				
	per doubling	♀: < 0.79 ♂: < 0.84	♀: 0.79-1.06 ♂: 0.84-1.15	♀: 1.07-1.41 ♂: 1.15-1.74	♀: 1.41-2.38 ♂: 1.74-3.31	♀: > 2.38 ♂: > 3.31
No. of cases	503	101	113	96	90	103
Person-years	50.452	10.079	10.188	10.066	10.067	10.051
Model 1	1.05 (0.97-1.13) P=0.258	0.98 (0.74-1.28) P=0.858	1.08 (0.83-1.41) P=0.567	0.93 (0.70-1.23) P=0.611	0.87 (0.66-1.16) P=0.346	1.0 (reference)
Model 2	1.04 (0.96-1.12) P=0.342	0.83 (0.63-1.10) P=0.192	1.03 (0.78-1.34) P=0.858	0.87 (0.66-1.16) P=0.341	0.91 (0.69-1.21) P=0.531	1.0 (reference)
24-h urinary sulfate excretion (mmol/24 h)						
		Gender-stratified quintiles				
	per doubling	♀: < 10.0 ♂: < 12.8	♀: 10.0-12.8 ♂: 12.8-16.2	♀: 12.8-15.3 ♂: 16.2-19.7	♀: 15.4-18.6 ♂: 19.7-23.9	♀: > 18.6 ♂: > 23.9
No. of cases	504	131	102	102	82	87
Person-years	50.539	9357	10.120	10.155	10.506	10.400
Model 1	0.87 (0.76-0.93) **P=0.039**	1.67 (1.27-2.19) **P<0.001**	1.20 (0.91-1.60) P=0.203	1.20 (0.90-1.60) P=0.212	0.93 (0.69-1.26) P=0.649	1.0 (reference)
Model 2	0.89 (0.77-1.03) P=0.107	1.04 (0.78-1.39) P=0.781	0.82 (0.61-1.10) P=0.193	0.96 (0.69-1.23) P=0.557	0.79 (0.59-1.07) P=0.132	1.0 (reference)

Model 1: crude, Model 2: model 1, adjusted for potential confounders (sex, age, BSA, history of cardiovascular disease, diabetes, current smoking, consumption of more than 1 alcoholic beverage per day)
HR; hazard ratio, CI; confidence interval, BSA; body surface area

Table 6: Cox proportional hazard models of the association of 24-h urinary thiosulfate and sulfate excretion and potential confounders with all-cause mortality

24-h urinary thiosulfate excretion (µmol/24 h)						
		Gender-stratified quintiles				
	per doubling	♀: < 0.79 ♂: < 0.84	♀: 0.79-1.06 ♂: 0.84-1.15	♀: 1.07-1.41 ♂: 1.15-1.74	♀: 1.41-2.38 ♂: 1.74-3.31	♀: > 2.38 ♂: > 3.31
No. of cases	445	109	102	84	68	82
Person-years	53.733	10.699	10.923	10.737	10.696	10.678
Model 1	0.93 (0.86-1.01) P=0.100	1.30 (0.97-1.73) P=0.078	1.19 (0.89-1.59) P=0.239	1.01 (0.74-1.37) P=0.962	0.83 (0.60-1.14) p=0.243	1.0 (reference)
Model 2	0.94 (0.86-1.02) P=0.143	1.06 (0.79-1.42) P=0.708	1.10 (0.82-1.49) P=0.502	0.91 (0.67-1.24) P=0.545	0.86 (0.62-1.18) P=0.350	1.0 (reference)
24-h urinary sulfate excretion (mmol/24 h)						
		Gender-stratified quintiles				
	per doubling	♀: < 10.0 ♂: < 12.8	♀: 10.0-12.8 ♂: 12.8-16.2	♀: 12.8-15.3 ♂: 16.2-19.7	♀: 15.4-18.6 ♂: 19.7-23.9	♀: > 18.6 ♂: > 23.9
No. of cases	445	151	90	81	69	54
Person-years	53.847	10.324	10.766	10.817	10.943	10.999
Model 1	0.65 (0.57-0.74) **P<0.001**	3.06 (2.25-4.18) **P<0.001**	1.72 (1.23-2.41) **P=0.002**	1.54 (1.09-2.17) **P=0.014**	1.29 (0.91-1.84) P=0.159	1.0 (reference)
Model 2	0.73 (0.63-0.84) **P<0.001**	1.48 (1.07-2.06) **P=0.019**	0.98 (0.69-1.38) P=0.896	1.04 (0.73-1.47) P=0.831	1.03 (0.72-1.47) P=0.892	1.0 (reference)

Model 1: crude, Model 2: model 1, adjusted for potential confounders (sex, age, BSA, history of cardiovascular disease, diabetes, current smoking and consumption of more than 1 alcoholic beverage per day)
HR; hazard ratio, CI; confidence interval, BSA; body surface area

Table 7: Cox proportional hazard models of the association of 24-h urinary sulfate excretion with all-cause mortality, causal path analysis

24-h urinary sulfate excretion†		
Model	HR (95% CI)	P-value
1	0.73 (0.63-0.84)	**<0.001**
2	0.75 (0.65-0.86)	**<0.001**
3	0.72 (0.63-0.83)	**<0.001**
4	0.75 (0.65-0.86)	**<0.001**
5	0.73 (0.65-0.86)	**<0.001**

*†HR per doubling *Skewed data, normalized by logarithmic transformation*

Model 1: adjusted for potential confounders (sex, age, BSA, history of cardiovascular disease, diabetes, current smoking, consumption of more than 1 alcoholic beverage per day), Model 2: model 1, additionally adjusted for hemodynamic parameters (SBP, DBP, heart rate and anti-hypertensive treatment), Model 3: model 1, additionally adjusted for lipid profile (total cholesterol, HDL and triglycerides, lipid lowering treatment), Model 4: model 1, additionally adjusted for hs-CRP, Model 5: model 1, additionally adjusted for eGFR

HR; hazard ratio, CI; confidence interval, BSA; body surface area, BMI; body mass index, SBP; systolic blood pressure, DPB; diastolic blood pressure, HDL; high-density lipoprotein, hs-CRP; high sensitivity C-reactive protein, eGFR; estimated glomerular filtration rate

3 Discussion

In this large cohort of individuals from the general population 24-h urinary sulfate excretion was found to be inversely associated with risk of CV events and all-cause mortality. While the association with all-cause mortality remained significant after adjustment for potential confounding factors, the association with CV events did not. This may create the impression that sulfate excretion is not directly connected to CVD and related mortality. However, studies on renal transplant recipients(45) and CHF patients(18) have shown urinary sulfate excretion to be associated with a beneficial CV risk profile and patient survival. It should also be noted that the definition of a CV event is limited to acute incidents, whereas CV causes of death also include chronic forms of CVD, such as CHF. Nevertheless, the association of sulfate excretion with death of a CV cause, found in our study, also lost its statistical significance on correction for potential confounders. As of yet, this unexpected finding is without explanation.

24-h urinary thiosulfate excretion, in turn, was not found to be associated with either risk of CV events or all-cause mortality, although Kaplan Meier analysis did show significantly different survival distributions for quintiles of thiosulfate excretion. This is in contrast with previous findings in the before mentioned high-risk population of renal transplant recipients, which, apart from sulfate, also link thiosulfate to a favorable CV risk profile and patient survival(45). The discrepancy may indicate that thiosulfate excretion is triggered in disease conditions, precluding determination of an association with baseline values in our cohort of predominantly healthy individuals from the general population. This hypothesis is substantiated by the increase in urinary thiosulfate excretion found in renal transplant recipients compared to healthy controls(45). Furthermore, whereas sulfate is an end-product, thiosulfate is an intermediate metabolite and therefore less stable(15). In fact, several studies have demonstrated reconversion of thiosulfate into H_2S(16, 24, 27, 48). Possibly, its dynamic nature provides another explanation for the absence of a robust relationship between urinary thiosulfate excretion and CV events or mortality, especially in a heterogeneous group of subjects from the general population. Besides, thiosulfate treatment has recently been shown

to offer protection in experimental models of hypertensive cardiac(37) and renal(38) disease, as well as diabetes(25). In the latter, this has been related to the activation of thiosulfate sulfurtransferase(25).

Several factors may underlie the inverse association between 24-h urinary sulfate excretion and all-cause mortality. For one thing, dietary intake of sulfate and the SAAs methionine and cysteine is known to augment urinary sulfate excretion(21, 22, 33). In the present study this is confirmed by the strong association with 24-h urinary urea excretion, which roughly reflects dietary protein intake. Diets high in protein or specific SAAs have been associated with decreased risk of CVD and mortality(19, 29, 31, 32, 47). However, results have been inconsistent(19, 29, 31, 32, 44, 47). Here, adjustment for 24-h urinary urea excretion on top of potential confounders had no effect on the statistical significance of the association between urinary sulfate excretion and all-cause mortality. This is in line with previous studies of renal transplant recipients and patients with diabetes mellitus type 1 nephropathy in which the association of sulfate excretion with all-cause mortality or renal disease progression was shown to be independent of dietary protein intake(1, 45).

Perhaps the degree of enzymatic production of sulfate from SAAs is a stronger determinant. This process links urinary sulfate excretion to H_2S, which is an intermediate in the transsulfuration pathway(15). Also, H_2S from other sources - including the reduction of bound sulfur and the reduction of sulfate by bacteria in the gut - contributes to the overall sulfate production(12, 34). Thus, urinary sulfate excretion may, at least in part, reflect endogenous production of H_2S, which is of particular interest as this gaseous signaling molecule is known to be involved in CV (patho)physiology(30). In experimental models, exogenous administration of H_2S or H_2S donors has been shown to protect against atherosclerosis(3, 23, 30), cardiac injury(36, 37, 42), renal disease(17, 37), and stroke(35, 50). Clinically, reduced plasma levels of H_2S-related metabolites have been associated with obesity and diabetes mellitus type 2(51). Research has shown H_2S to be involved in blood pressure regulation(54), lipid metabolism(13, 52), inflammation(40) and renal function(17). However, in causal path analysis, adjustment for associated variables did not identify any of these factors as the underlying mechanism for the association between urinary sulfate excretion and all-cause mortality.

In our study, subjects from the highest quintile of sulfate excretion did show the highest eGFR. This is in accordance with results from previous studies on the relationship of sulfate (excretion) and renal function(1, 9, 45, 46). As eGFR itself is strongly associated with a reduced risk of CVD and all-cause mortality(4) one may assume this explains the inverse association of urinary sulfate excretion with all-cause mortality found in our study. However, as mentioned above, adjustment of this association for eGFR on top of potential confounders had no effect on its statistical significance. Besides, under steady-state conditions the urinary excretion of any metabolite is not determined by renal function, but rather represents its metabolic turnover which has to be matched to avoid systemic accumulation. Subgroup analysis revealed a lower HR for subjects with an eGFR below 60 ml/min compared to those with normal renal function. Although the interaction was only borderline significant, the observed trend substantiates the notion that urinary sulfate excretion is not a reflection of renal function.

Interestingly, in CHF patients both urinary excretion and clearance, but not the plasma concentration of sulfate have been found to be associated with favorable disease outcome(18). Collectively, these findings suggest that the renal handling of sulfate is of importance. Unfortunately, in the present study sulfate clearance could not be assessed.

Subgroup analysis also uncovered a lower HR for subjects with normal cholesterol levels compared to those with dyslipidemia. The interaction between cholesterol levels and the association of sulfate excretion with all-cause mortality is likely connected to the process of steroid sulfation. Sulfate conjugation increases hydrophilicity and thereby promotes urinary excretion of steroids, but also modulates cholesterol function(6). Possibly, an increased need for sulfation explains the relatively small advantage of sulfate excretion in subjects with dyslipidemia.

Considering the before mentioned evidence from previous studies of high-risk populations, the trend towards an interaction between the presence of diabetes and the association of sulfate excretion with all-cause mortality is surprising. Unexpectedly, the association appeared to be most pronounced for subjects without diabetes, the meaning of which remains to be elucidated.

This study has several limitations. Firstly, it was carried out in a single center and includes almost only Caucasian subjects. Consequently, our results may not be representative for other populations. Also, our data are observational and therefore do not allow causality of the relationship between urinary sulfate excretion and all-cause mortality to be established. Furthermore, 24-h urinary urea excretion provides only a rough estimate of dietary protein intake and is therefore inferior to actual dietary information, which unfortunately was not available. Strengths of our study include the large number of subjects and their extensive characterization, the use of 24-h urine samples and the long duration of follow-up.

In conclusion, 24-h urinary sulfate, but not thiosulfate excretion is inversely associated with all-cause mortality in a large, general population based cohort, in particular in subjects with normolipidemia. Whether functional properties of sulfate itself or rather its relationship with H_2S underlies this association remains to be elucidated. Accordingly, further research is warranted to unravel the role of sulfate and its urinary excretion in physiology and disease.

4 Innovation

Urinary sulfate excretion and health benefit go hand in hand, not only in high-risk patient groups, but also in the general population. The strong inverse association between urinary excretion of sulfate and all-cause mortality in a large, general population based cohort emphasizes the (patho)physiological importance either of sulfate itself or of its precursor hydrogen sulfide. Accordingly, sulfate excretion holds promise as a marker of physiological disturbance and may even serve as a target for nutritional intervention to promote healthy aging.

5 Materials and methods

5.1 Study population

Data on subjects of the Prevention of Renal and Vascular End-stage Disease (PREVEND) study were used for this analysis. The PREVEND study was designed to prospectively investigate the natural course of albuminuria and its relation to renal and cardiovascular disease in a large cohort drawn from the general population, and has previously been described in detail(14). In brief, from 1997 to 1998 a total of 85421 inhabitants of Groningen, the Netherlands were approached with a short questionnaire (regarding demographics, medication use, and pregnancy) and a vial to collect an early morning urine sample. Pregnancy and type-1 diabetes were exclusion criteria. In 40856 responding subjects urinary albumin concentration was determined. Subsequently, 6000 participants with a urinary albumin concentration ≥ 10 mg/l and 2592 randomly selected subjects with a urinary albumin concentration < 10 mg/l enrolled in the study. In total, the PREVEND cohort consists of 8592 individuals. The study was approved by the medical ethics committee of the University of Groningen and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. For the present analysis, urine samples from the second survey (in 2001 to 2003) were selected and this time-point was considered baseline. Due to unavailability of urine samples or data on urine volume 16 of 6855 potential subjects had to be excluded. Of the remaining 6839 participants - aged 32 to 80 years - 967 (14.2%) had albuminuria, whereas 5854 (85.8%) did not. For 18 participants (0.3%) this data was missing.

5.2 Data collection

All subjects of the PREVEND study visited the outpatient research unit twice for baseline investigation. At the first visit, participants filled out a questionnaire on demographics, general health, CVD history, medication, smoking habits and alcohol consumption. Height, weight and waist circumference were measured. In addition, a fasting blood sample was collected and stored at -80 °C until analysis. At the second visit, blood pressure was measured in supine position every minute for 8 minutes with an automatic Dinamap XL Model 9300 series device (Johnson-Johnson Medical, Tampa, FL), and the mean of the last 2 recordings was listed. Additionally, participants collected 24-h urine after oral and written instructions. Urine samples were stored at -20 °C until analysis.

5.3 Laboratory measurements

Serum total cholesterol, serum glucose and serum and urinary creatinine were measured by Kodak Ektachem dry chemistry (Eastman Kodak, Rochester, NY). High-density lipoprotein (HDL) cholesterol was determined with a homogenous method (direct HDL, Aeroset TM System, Abbott Laboratories, Abbott Park, IL). Triglycerides were measured enzymatically.
Urinary albumin concentrations and high sensitivity C-reactive protein (hs-CRP) were determined by nephelometry (Dade Behring Diagnostics, Marburg, Germany). Urinary urea was measured with a MEGA clinical chemistry analyzer (Merck, Darmstadt, Germany) by a photometric test with the urease-GIDH method. Urinary sulfate was determined by ion

exchange chromatography (type 861; Metrohm, Herisau, Switzerland), using a Metrosep A Supp 4 - 250/4.0 column. Intra- and inter-assay variations were 2.0% and 4.3%, respectively. Urinary thiosulfate was measured by reverse-phase HPLC as previously described(26, 45). In brief, 25 µl of urine was derivatized with 5 µl of 46 mM monobromobimane, 25 µl of acetonitrile and 25 µl of 160 mM HEPES/16 mM EDTA pH 8 buffer (Invitrogen, Carlsbad, CA) for 30 min. Derivatization was stopped by addition of 50 µl of 65 mM methanosulfonic acid (Fluka, Buchs, Switzerland), and proteins were removed by re-centrifugation. Intra- and inter-assay variations were 8.6% and 9.3%, respectively.

5.4 Definitions and primary outcome definition

Body surface area (BSA) was defined following the Dubois & Dubois formula: 0.007184 x ($Height^{0.725}$ x $Weight^{0.425}$)(7). Diabetes mellitus was defined according to the guidelines of the American Diabetes Association as a fasting glucose level ≥ 7.0 mmol/l and/or the use of anti-diabetic medication(43). Estimated glomerular filtration rates (eGFR) were calculated following the Chronic Kidney Disease Epidemiology collaboration equation (CKD-EPI) formula(20). Urinary concentrations of creatinine, albumin, thiosulfate, and urea were multiplied by the 24-h urinary volume to determine 24-h urinary excretion. Information on death, cardiovascular disease and hospitalization for cardiovascular disease was obtained from the participant's questionnaire and the Dutch national registry of all hospital discharge diagnoses (Prismant). Data were coded according to the International Statistical Classification of Diseases (ICD-10) and the International Classification of Health Interventions(11). All-cause mortality was set as the primary endpoint and cardiovascular events were set as the secondary endpoint of this study.

5.5 Statistical analyses

Statistical analyses were performed with the Statistical Package for Social Sciences (SPSS Statistics, IBM Corporation, Armonk, NY) version 22.0, STATA/SE software (Release 13; StataCorp, College Station, TX) and R version 3.0.1 (Vienna, Austria). Graphs were drawn in GraphPad Prism (version 5.0, GraphPad Software, La Jolla, California, USA).

Distributions of variables were visualized with histograms and Q-Q plots. Normally distributed continuous data are presented as mean ± standard deviation (SD). Skewed data are presented as median (interquartile range IQR) and were normalized prior to analyses by logarithmic transformation (triglycerides, glucose, hs-CRP, and 24-h urinary albumin, sulfate, and thiosulfate excretion). 24-h urinary thiosulfate and sulfate excretion values were log-transformed according to the base of two, to allow interpretation of hazard ratios (HRs) per doubling. Nominal data are presented as *n* (%).

Differences in baseline characteristics between groups were determined using One-way ANOVA for normally distributed continuous data, the Kruskal-Wallis test for skewed continuous data, and the Chi-square test for nominal data. Chi-square tests were also used to compare the distributions of quintiles of 24-h urinary thiosulfate and sulfate excretion among survivors and non-survivors.

To compare the survival distributions for quintiles of 24-h urinary thiosulfate and sulfate excretion Kaplan-Meier plots and log-rank tests were applied. Follow-up time was defined as the period from the date of urine collection until the date of death or the end of follow-up (on January 1, 2009).

Univariable linear regression analyses were performed for 24-h urinary thiosulfate and sulfate excretion and potential confounding factors, followed by multivariable analyses with backward selection. Subsequently, the association between 24-h urinary thiosulfate and sulfate excretion and potential intermediary factors was determined using univariable linear regression analyses.

As several subjects showed missing values for one or more baseline variables and bias can be introduced when subjects with missing values are excluded, multiple imputation was used to obtain 5 imputed datasets. Using the imputed dataset, Cox proportional hazards analysis was applied to study the association between 24-h urinary thiosulfate and sulfate excretion and risk of CV events and all-cause and CV mortality. Follow-up time was defined as the period from the date of urine collection until the date of a CV event, death or the end of follow-up (on January 1, 2009). Participants were censored if they moved to an unknown destination or, in the case of CV events, if they died of a non-CV cause. HRs are reported with 95% confidence intervals (95% CI). Cox regression analysis with restricted cubic splines with 3 knots was used to test for potential non-linearity of the associations of 24-h urinary thiosulfate and sulfate excretion with all-cause mortality. Finally, Cox proportional hazards analysis with adjustment for potential confounders was applied to assess HRs for the association of 24-h urinary sulfate excretion with all-cause mortality across various subgroups.

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Conflicts of interest

None declared.

Abbreviations

BMI; body mass index
BSA; body surface area
CI; confidence interval
CKD-EPI; Chronic Kidney Disease Epidemiology collaboration equation
CSE; cystathionine γ-lyase
CV; cardiovascular
CVD; cardiovascular disease
DPB; diastolic blood pressure
EDTA; Ethylenediaminetetraacetic acid
eGFR; estimated glomerular filtration rate
HDL; high-density lipoprotein
HEPES; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR; hazard ratio
hs-CRP; high sensitivity C-reactive protein
H_2S; hydrogen sulfide
ICD-10; International Statistical Classification of Diseases
IQR; interquartile range
PREVEND: Prevention of Renal and Vascular End-stage Disease
SBP; systolic blood pressure
SD; standard deviation

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